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Catalytic function of avian cytochrome P450 1A4 and 1A5 (CYP1A4 and CYP1A5) enzymes heterologously expressed using *in vitro* yeast system

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**Catalytic function of avian cytochrome P450 1A4 and 1A5
(CYP1A4 and CYP1A5) enzymes heterologously expressed
using *in vitro* yeast system**

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Abstract

The present study clarifies the enzymatic properties of two avian cytochrome P4501A (CYP1A) paralogs, CYP1A4 and 1A5, using a yeast-based vector system. Recombinant CYP1A4 and 1A5 proteins from common cormorant (*Phalacrocorax carbo*) were expressed in yeast cells, and showed typical reduced CO-difference spectra with a peak at 446 nm. Kinetic analysis of *O*-dealkylase of methoxy-, ethoxy-, pentoxy- and benzyloxyresorufin catalyzed by the CYP1A enzymes revealed that $V_{\max}$ value for ethoxyresorufin *O*-deethylase (EROD) activity was much higher than that for the other three *O*-dealkylase activities for both isozymes. Interestingly, remarkable substrate specificity of the CYP1As was observed for *O*-dealkylation of benzyloxyresorufin and methoxyresorufin;

CYP1A4 was highly specific for catalyzing benzyloxyresorufin *O*-debenzylase activity, whereas CYP1A5 was more efficient in catalyzing methoxyresorufin *O*-demethylase activity. The present study also measured CYP1A-dependent EROD activity in the presence of 2,3,7,8-tetrachlorodibenzofuran (TCDF) to evaluate the ability of this dioxin-like congener to inhibit the EROD activity. One hundred nM TCDF noncompetitively inhibited CYP1A5-dependent EROD activity, although no inhibitory effect was detected for CYP1A4-dependent EROD activity. These results indicate that the avian CYP1A paralogs have different affinities for substrate and inhibitor, thus suggesting their distinct physiological and toxicological roles.

Keywords: CYP1A4; CYP1A5; Birds; Heterologous expression; EROD; Biomarker; 2,3,7,8-tetrachlorodibenzofuran

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Expression of members of the cytochrome P4501A (CYP1A) subfamily is inducible by environmental contaminants such as dioxin and related compounds (DRCs). It is generally

known that the induced CYP1A is involved in the disruption of the signaling pathway regulated by endogenous substrates and the production of reactive oxygen species, thus indicating that CYP1A induction by xenobiotics is linked with the toxic effects of environmental contaminants.

Our recent study (Kubota et al., 2006) revealed that the common cormorant (*Phalacrocorax carbo*) possesses at least two paralogous CYP1A genes that are orthologous to chicken (*Gallus gallus*) CYP1A4 and 1A5. Furthermore, we have clarified that cormorants collected from Lake Biwa in Japan accumulate high levels of DRCs in the liver and induce CYP1A4 and 1A5 at transcriptional and translational levels (Kubota et al., 2004, 2005, 2006). While extensive data are available on mammalian CYP1A1 and 1A2, enzymatic properties of CYP1A4 and 1A5 isozymes in avian species have not been clarified until now. Therefore, the present study investigated enzymatic properties of avian CYP1A4 and 1A5 that were heterologously expressed in yeast cells.

The full-length cDNAs of common cormorant CYP1A4 and 1A5 were isolated from the hepatic cDNA library (Kubota et al., 2006). The CYP1A4 and 1A5 were expressed with plasmids containing cDNA of each isozyme in an *in vitro* yeast expression system following the method of Chung et al. (2004). Expression of recombinant CYP1A proteins in yeast microsomes was confirmed by the reduced CO-difference spectrum and detected by western blotting. Kinetics and specificity of the CYP1A enzymes toward *O*-dealkylation of methoxy-, ethoxy-, pentoxy- and benzyloxyresorufin (MROD, EROD, PROD and BROD, respectively)

were investigated as described by Kubota et al. (2005) with some modifications.

Yeast microsomes containing each common cormorant CYP1A enzyme showed quantifiable Soret maximum at 446 nm. The concentrations of CYP1A4 and 1A5 proteins were estimated to be 351 and 275 pmol/mg protein, respectively. By immunoblotting using an anti-rat CYP1A1 polyclonal antibody, a single cross-reactive band was detected in the yeast microsomes containing CYP1A4 or 1A5.

Kinetic analysis of AROD activity catalyzed by the recombinant CYP1A enzymes revealed that the AROD reaction follows typical Michaelis Menten kinetics. Among all the AROD activities, $V_{\max}$ value of EROD activity was the highest for both isozymes ($V_{\max} = 1.0$ and 1.5 nmol/min/nmol CYP for CYP1A4 and 1A5, respectively). Regarding BROD activity, CYP1A4 ($V_{\max} = 0.98$ nmol/min/nmol CYP) was more specific than CYP1A5 ($V_{\max} = 0.028$ nmol/min/nmol CYP). As for MROD activity, CYP1A5 (0.89 nmol/min/nmol CYP) exhibited a greater capacity than CYP1A4 (0.095 nmol/min/nmol CYP). On the other hand, both CYP1A4 and 1A5 showed relatively weak $V_{\max}$ values for PROD activity with 0.10 and 0.15 nmol/min/nmol CYP, respectively. Although the activities reported for the two recombinant CYP isozymes under the conditions present in a yeast background may not reflect enzyme activity *in vivo*, the clear difference in AROD profiles between the avian CYP1A paralogs implies that the two CYP1As have distinct potential for substrate recognition.

CYP1A4- or 1A5-dependent EROD activity was measured in the presence and absence of

TCDF to examine the inhibitory effect of this DRC congener. The results showed that CYP1A5-dependent EROD activity was specifically inhibited by TCDF, whereas no inhibition was observed for CYP1A4-dependent EROD activity (Fig. 1). Considering that treatment with TCDF lowered the maximum CYP1A5 activity ($V_{\max}$), but less affected the K_m (0.152 μ M and 0.169 μ M with or without addition of TCDF, respectively), the mode of inhibition by TCDF is regarded to be noncompetitive [inhibitor constant (K_i) = 74 nM]. The noncompetitive inhibition indicates that TCDF does not affect binding of ethoxyresorufin to CYP1A5, and binds CYP1A5 at a site distinct from binding site of ethoxyresorufin. Several previous investigations have reported that the catalytic function of rodent CYP1A2 was inhibited by certain DRCs (Staskal, et al., 2005). These results clearly indicate that TCDF also inhibits the enzymatic activity of avian CYP1A5, which supports the previous hypothesis that the avian CYP1A5 is orthologous to mammalian CYP1A2 (Goldstone and Stegeman, 2006; Kubota et al., 2006).

Further study is necessary to understand physiological and toxicological roles of avian CYP1A4 and 1A5. The approach using recombinant CYP proteins may provide a novel insight into risk assessment of environmental contaminants in avian species.

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Figure caption:

Fig. 1. Effects of TCDF (100 nM) on EROD activities catalyzed by recombinant common cormorant CYP1A4 (a) and 1A5 (b). Inhibitor constant (K_i) was calculated by the equation, $V_{\max}^{\text{app}} = V_{\max}/(1 + [I]_0/K_i)$. Nonlinear regression was used for the determination of kinetic parameters. Triangles and circles indicate initial velocities in the presence and absence of TCDF, respectively. Data are represented by means of duplicate assays. Each assay was conducted in triplicate and variation of data among triplicate analyses was within 20%.

Fig. 1.